FLOW CYTOMETRIC EVALUATION OF ACROSOME FUNCTION/DYSFUNCTION IN THE STALLION

A Thesis

by

TEGAN S. BOSARD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2006

Major Subject: Veterinary Medicine

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Approved by:

Co-Chairs of Committee,	Charles C. Love
	Dickson D. Varner
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ABSTRACT

Flow Cytometric Evaluation of Acrosome Function/Dysfunction

in the Stallion. (August 2006)

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Co-Chairs of Advisory Committee: Dr. Charles C. Love
Dr. Dickson D. Varner

The objective of this study was to establish a rapid and efficient assay that would assess acrosomal status and function of the stallion acrosome. Ejaculates from fertile and subfertile stallions were extended to $25x10^6$/mL and divided into aliquots (1mL) treated with no ionophore (control) or 10µM A23187 and incubated at 37ºC for 0, 1, 2, and 3h. Following incubation, samples were fixed with 2% paraformaldehyde for 10 minutes at room temperature; then stored at 4°C in Dulbecco's Phosphate-buffered saline (DPBS) for 0, 24, and 72 hours (i.e. post-fixation storage). After post-fixation storage samples were then permeabilized with 95% ethanol at -20ºC for 10 minutes. Samples were resuspended in 20% fetal bovine serum in DPBS, labeled with fluorescein isothiocyanate for 10 minutes, and analyzed by flow cytometry.

Post-fixation storage produced fewer ($P<0.05$) acrosome intact (AI) spermatozoa and a higher ($P<0.05$) fluorescence intensity than respective fresh samples. Regardless of incubation time or treatment, cool-stored samples averaged ~6% lower ($P<0.001$) AI spermatozoa than the corresponding fresh semen; however, cooled storage did not alter ($P>0.2$) the overall fluorescence

properties as compared to fresh semen (730±8.08 vs. 734±8.01 fluorescence intensity units, respectively). For fertile stallions, the percentage of AI spermatozoa was higher ($p<0.01$) in control samples than A23187 samples at incubation times 1, 2, and 3h (Control-59, 56, and 51% vs. A23187- 46, 29, and 23%, respectively), but not at Time 0. For subfertile stallions, the percentage of AI spermatozoa was not affected by ionophore treatment ($P>0.05$) or incubation period ($P>0.05$).

The results suggest that post-fixation storage in DPBS for up to three days is still representative of the acrosomal competence of the original sample. In addition, spermatozoa stored for 24 hours in an Equitainer™ exhibited a small (~6%) but significant decrease in the percentage AI spermatozoa. Storage conditions may therefore, affect acrosomal integrity and contribute to reduced fertility when cooled-semen is used. Subfertile stallions exhibited little response [<11% acrosome reacted (AR)] after 3h of A23187 exposure, while the fertile stallions demonstrated a substantial response (≥ 36% AR) as soon as 1h after ionophore exposure. This assay diagnosed acrosomal dysfunction in stallions with unexplained subfertility.

DEDICATION

I dedicate this work to:

My husband: Christopher Lutrick,

My parents: Scott and Suzan Bosard,

&

My family and friends.

These loved ones provided me stable ground to build my dreams higher than I ever imagined. Without them I would not be woman I am today. Mom and Dad, thank you for ALWAYS affording me with everything I needed to succeed.

ACKNOWLEDGEMENTS

I would like to thank Drs. Varner, Love, and Brinsko for their guidance throughout my research. Dr. Varner, I greatly appreciate all the opportunities you provided me to help me achieve my goals. Dr. Brinsko, thank you for your frequent inquisitions, it has made me a better student. Dr. Love, your time, consideration, and patience provided me the fortitude to understand flow cytometry and complete my project. For the charitable hours, cubby-hole companionship, and pipetting techniques of Jenny Spooner, Thank you! I would like to thank Dr. Johnson and Vince Hardy for allowing me to invade their laboratory for paraformaldehyde preparation. Thanks to Dr. Smith for his patience and providing me with the tools to answer my questions about flow cytometry. I appreciate the collaborative efforts of the TAMU College of Veterinary Medicine, Equine Theriogenology Laboratory for helping me with collections. I feel privileged to have met and worked with all of you, Thank you!

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INTRODUCTION

Routine breeding soundness examinations do not always reveal the underlying cause of reduced fertility. Historically, motility has been used as a laboratory measure of spermatozoal function [1,2,3,4]. The evaluation of other spermatozoa characteristics has provided additional criteria for assessment of semen quality in stallions. These include spermatozoa morphology [4,5,6], chromatin structure [6,7], and membrane viability [8]. Amann and Hammerstedt [9] reported that assessment of only one sperm characteristic could result in overestimation of fertility. These workers suggest that each additional sperm characteristic measured will create a more accurate representation of an individual's fertility. Additionally, Wilhem et al. [10] demonstrated that the combination of sperm motility, viability, and penetration rates into zona-free hamster oocytes explained 72% of the variability in fertility among stallions. These studies demonstrate the value of analyzing multiple sperm characteristics to predict the fertility of stallions.

Reduced fertility can be related to acrosomal dysfunction. Meyers et al. [11] reported that progesterone-induced acrosome reactions were lower in subfertile than fertile stallions. In another study, Meyers et al. [12] reported that stallion semen with low spermatozoal motility and morphology contained fewer

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This thesis follows the style of Theriogenology.

($P<0.05$) zona pellucida bound sperm and produced less ($P<0.05$) zona pellucida-induced acrosome reactions than fertile stallions. Varner et al. [13] reported that acrosomal dysfunction could be a cause of reduced fertility in stallions that otherwise display normal semen parameters. In this study, sperm from subfertile stallions had a dramatically reduced incidence of acrosome reactions following calcium ionophore (A23187) challenge, even though the spermatozoal populations from these stallions possessed normal motility and morphologic parameters. This finding demonstrated that a single defect in the acrosome could result in subfertility.

The status and function of the stallion acrosome is difficult to evaluate clinically. Clinical evaluation of the acrosome of stallions has been limited to light [14], fluorescence [11,13,14,15], and electron [13,14] microscopic techniques. Light microscopy provides a natural view of the sperm, yet does not enable the viewer to appreciate the compartments of the sperm. Fluorescence microscopy permits vivid visualization of appropriately labeled acrosomes, but can be laborious to perform and the evaluation is restricted to a relatively small number (hundreds) of sperm. Transmission electron microscopy permits good visualization of acrosomal membranes; however this technique is both expensive and time consuming, with the ability to examine only a few sperm (50-100). In contrast, flow cytometric techniques allow evaluation of fluorescence intensity changes within a larger population (thousands) of sperm. Cytochemical labels have been developed to assess acrosomal response to

various *in vitro* treatments [8,11,15,16,17,18,19], but few studies have addressed the clinical relevance of acrosome reaction rates to fertility.

Incorporation of a battery of tests that analyze a realm of sperm characteristics and compartments should provide researchers and clinicians a more accurate estimation of the fertilizing potential of semen samples. Because of the importance of acrosomal function to fertilization, development of an assay that can quickly and reliably evaluate acrosomal integrity and functionality would be a meaningful component of the breeding soundness examination. Ideally, the assay should accurately describe the basal state of the acrosome (intact vs. damaged/reacted) and should also determine its functional status (ability to react). The assay should be adaptable to semen collected at remote locations for transport to a reference laboratory to accommodate field collections, as well as be applicable to freshly collected, cool-transported, or cryopreserved semen.

The present study investigates the use of flow cytometric techniques to assess sperm acrosome status and integrity in fertile and subfertile stallions, as well as evaluates the effect of cooled storage on acrosomal characteristics.

OBJECTIVES

The objectives of this study are to determine the effect of fixation and duration of storage at 4ºC on acrosomal status and function of stallion semen. I also want to detect the effects of 24 hour cooled-storage (~4ºC) in Equitainer™ transport containers on acrosomal status and function, as well as determine differences in acrosomal status and function between fertile and subfertile stallions.

LITERATURE REVIEW

Sperm Preparation for Fertilization

Sperm Maturation

The mammalian spermatozoon consists of specific structures that prepare it for travel through the female reproductive tract and for oocyte interaction. Unlike lower vertebrates and invertebrates, mammalian sperm must undergo multiple changes after leaving the testis to gain the capacity to fertilize an oocyte; hence, the term capacitation [20]. Sperm gain progressive motility and the ability to fertilize as they pass through the epididymis, attaining full fertilizing capability by the time they reach the proximal cauda epididymis [20,21]. Mammalian sperm also experience modifications of both integral and surface plasma membrane glycoproteins during epididymal maturation [22,23]. Modifications to protein distribution and protein expression, as well as lipid and protein substitutions also occur during this maturation period [20]. Membrane alterations occur not only in the plasma membrane overlying the head and the principal piece, but in the outer acrosomal membrane and are presumed to prevent premature capacitation and stimulation of the acrosome reaction.

Capacitation

Sperm that have matured in the epididymis are motile; however they do not possess the capacity to fertilize immediately. The process by which a spermatozoon acquires the ability to fertilize is capacitation [24]. Epididymal maturation and ejaculatory secretions apparently modify the mammalian sperm's

plasma membrane proteins and lipid composition to prepare the spermatozoon for transport through the capacitating environment of the female's genital tract [20]. However, freshly ejaculated sperm must undergo post-ejaculation metabolic and membrane-associated changes (i.e., capacitation) to attain full fertilizing power [20,23,25,26,27].

The rearrangement of the plasma membrane through epididymal maturation and seminal fluid exposure provide decapacitation factors to prevent premature capacitation [23,28,29]. Decapacitation factors enable the sperm to be sustained within the female's genital tract to allow a time-delayed response to ensure maximal fertilizing ability at the site of fertilization. Seminal fluid is believed to contribute to decapacitation by stabilizing spermatozoal membranes to prevent membrane destabilization (ie. capacitation). It has been shown in men [30] and stallions [31] that the prostate gland secretes cholesterol rich prostasomes into seminal fluid to prevent cholesterol depletion and delay capacitation in the sperm, such that epididymal sperm (i.e. sperm not exposed to seminal fluid) are responsive to progesterone-induced acrosome reactions [29,32]. Ejaculated sperm are not responsive to progesterone until after the capacitation process is completed [11,23,32,33,34,35]. The prior studies support the fact that seminal fluid supplies decapacitation factors and somehow masks spermatozoal progesterone receptors to prevent premature induction of the acrosome reaction.

Capacitation of mammalian sperm includes the removal of the decapacitating factors, as well as the structural rearrangement of the sperm plasma membrane; exposure of the sperm's zona pellucida (ZP) receptors; and a change in flagellar beat pattern, termed hyperactivated motility [20,23,27]. Hyperactive motility enables sperm to maneuver through the cumulus cells and assist in ZP penetration to permit oocyte binding following the AR. Multiple changes occur in the sperm's plasma membrane during capacitation, such as the reordering of phospholipids and depletion of cholesterol. Spermatozoal plasma membranes have an asymmetric distribution of lipids between the inner and outer layers of the bilayer. This transbilayer asymmetry is maintained by an ATP-dependent aminophospholipid translocase, which is thought to be inhibited by the elevated intracellular calcium levels during capacitation in sperm [23,36,37]. The capacitation process leads to a depletion and translocation of lipids and removal of cholesterol from the plasma membrane of sperm [20,23,27]. Cholesterol efflux is believed to facilitate increased membrane fluidity, plasma membrane destabilization, tyrosine phosphorylation, and an increased intracellular pH [23,29,37,38]. It is believed that cholesterol is a major decapacitating factor and that plasma-membrane cholesterol concentrations determine capacitation rates [20,23,29,39]. Cholesterol concentration is a major variable of sperm between species, as well as between individuals [20,39]. The plasma membrane of stallion sperm contains relatively high cholesterol content (37%), as compared to other mammalian sperm (25%). This feature presumably

provides stallion sperm with a lengthened capacitation period, 8-6 hours, whereas species, such as boars and rams, with lower cholesterol content require only 1-2 hours for capacitation [20,23]. These differences in cholesterol/phospholipid ratios could serve as a potential source of variation of capacitation and/or acrosome reaction rates between stallions [40]. This could parallel the rate of physiological acrosome reaction, a lower cholesterol/phospholipid ratio enabling the acrosome to react sooner. It is believed that the changes that occur during capacitation play a critical role in preparing the sperm for the subsequent acrosome reaction [41].

Acrosome Reaction (AR)

The mammalian spermatozoon acrosome is a Golgi-derived secretory granule enclosed by an inner and outer acrosomal membrane. Acrosomal exocytosis of the mammalian sperm is the series of poorly defined events that result in the fusion and vesiculation [42] of the superficial plasma membrane with the acrosome's outer acrosomal membrane. The acrosome of the sperm overlies the nucleus at the apical region of the head, forming a cap-like structure over the nucleus (Figure 1, Image A). The most superficial membrane that covers the apical surface of the sperm head is the plasma membrane. Beneath the plasma membrane, localized at the apical region of the head, is the outer acrosomal membrane (OAM). Intermittent fusion between these two membranes, with resultant vesiculation and fenestration of the combined membranes, is termed the acrosome reaction. The acrosomal matrix, which

underlies the outer acrosomal membrane, contains both structural and non-structural (non-enzymatic and enzymatic) components. Fusion of the plasma membrane with the OAM (Figure 1, Image C) allows the release of nonadherent components of the acrosomal matrix to expose the inner acrosomal membrane (IAM) that covers the crown of the nuclear envelope, Figure 1, Image D. The head of the mammalian sperm is also divided into domains that contain different concentrations and distributions of plasma membrane constituents [42]. The region that overlies the acrosome is the pre-equatorial region, followed by the equatorial region at the terminus of the acrosome, and the post-equatorial region caudal to the acrosome (Figure 1, Image B). At the conclusion of the AR, the IAM becomes contiguous with the plasma membrane of the equatorial region, forming a hairpin structure. This hairpin is believed to be the site of sperm-oolemma interaction [20,23,42].

The AR of mammalian sperm is characterized by the release of proteolytic enzymes that enable ZP penetration and subsequent exposure of sperm-oolemma binding proteins on the IAM [23,42,43]. The specific physiologic interactions that occur during the AR are not completely understood; however, it is thought that receptors revealed through capacitation permit sperm-ZP binding which, in turn, initiates a signal cascade to stimulate a host of enzymatic reactions and elevate cytosolic calcium concentrations and pH, as diagramed in Figure 2 [44]. When the ZP binds to surface G_i-coupled (G_i) and

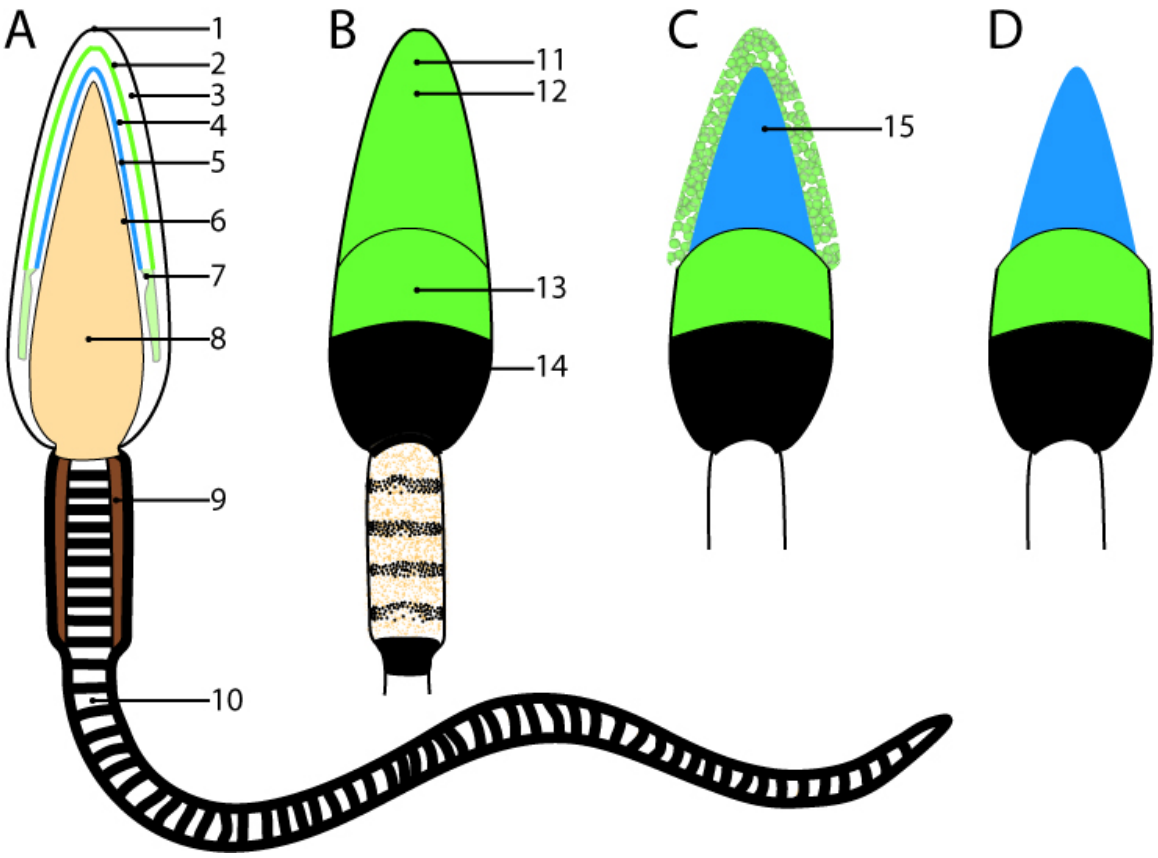

Figure 1. Diagrammatic representation of the stallion sperm. **Image A**: longitudinal section of the spermatozoon. 1. Plasma Membrane; 2. Outer acrosomal membrane; 3. Cytosol; 4. Acrosomal contents; 5. Inner acrosomal membrane; 6. Nuclear envelope; 7. Equator; 8. Nucleus; 9. Mid-piece; 10. Principle piece; **Image B**: Plasma membrane domains of the head. 11. Apical domain; 12. Pre-equatorial domain; 13. Equatorial domain; 14. Post-equatorial domain; **Image C**: Acrosome reaction, demonstrating the vesiculation of the plasma membrane with the outer acrosomal membrane. 15. Exposed inner acrosomal membrane. **Image D**: After the acrosome reaction, the inner acrosomal membrane remains.

tyrosine kinases (TK) receptors of the sperm, it is thought to initiate a signal-transduction cascade. The sperm plasma membrane receptors can also activate sodium (Na^+)/hydrogen (H^+) exchange mechanisms, thus creating an alkalization of the cytosol by the removal of H^+ and the depolarization of the plasma membrane [44,45]. Ligand-bound G_i-coupled receptors activate adenylate cyclase (AC) to produce cyclic AMP (cAMP), which activates protein

kinase A (PKA). Protein kinase A activates voltage-dependent Ca^{2+} channels of the OAM to release internal stores of calcium, permitting the first increase of cytosolic calcium concentrations [20,39,44]. It is thought that the small release of Ca^{2+} from internal stores acts synergistically with ZP-bound tyrosine kinase and G_i-coupled receptors to activate phospholipase Cγ (PLC_γ) and phospholipase $C\beta_1$ ($PLC_{\beta1}$) respectively [39,42,44]. Phospholipase enzymes (PLC β_1 and PLCγ) hydrolyze phosphotidylinositol 4-5 bisphosphate (PIP_2) in the sperm plasma membrane into inositol triphosphate (IP_3) and diacylglycerol (DAG). Diffusion of IP_3 to the OAM reinforces calcium release from acrosomal stores. Remaining localized around the cytosolic surface of the plasma membrane, DAG stimulates protein kinase C (PKC) which mediates protein phosphorylation to activate external calcium channels to open. Both IP_3 and DAG stimulate a positive feedback loop to increase cytosolic calcium concentrations between the plasma membrane and OAM [20,39,44,46]. Opening of external plasma membrane calcium channels activated by PKC allows a second larger surge of calcium. Protein kinase C also activates phospholipase A_2 (PLA_2) to simulate the hydrolysis of arachidonic acid (AA) within the plasma membrane. Arachidonic acid is converted to prostaglandins (PG) and leukotriens (LT) by cyclooxygenase (COX) and lipoxygenase (LOX). This mechanism is thought to provide another means for induction of the acrosome reaction. The rise of cytosolic ionic calcium to a supramicromolar concentration stimulates the activation of actin severing proteins, a stage

preceding plasma membrane and OAM fusion. As in somatic cells, actin severing proteins destroy the actin network (F-actin) of the cytoskeleton by filament fragmentation or end-blocking [47,48]. The F-actin breakdown allows the collapse of the cytoskeleton, permitting fusion of the plasma membrane with the underlying OAM [20,39,49,46]. Vesiculation of the plasma membrane with the OAM permits the release of acrosomal contents, primarily acrosin and hyaluronidase; thereby facilitating ZP penetration and spermatozoal entrance into the perivitelline space [43]. The acrosome reaction exposes oolemma-binding sites on the IAM of the sperm to allow oolemma interaction and the completion of fertilization.

Several steps may be required to complete the AR, so it is difficult to solely attribute the destruction of the cytoskeleton to a single messenger. Under *in vitro* conditions, actin severing proteins require an even higher Ca^{2+} concentration to react than that under *in vivo* conditions, suggesting that other factors may support actin filament demolition. Calcium and phosphorylated phosphatidylinositol lipids (PPIs) influence each other's signaling pathways [48]. It has been reported that PPIs, like PIP_2, strongly inhibit the severing function of gelsolin, a common Ca^{2+}- sensitive F-actin severing protein, whereas this is not the case for PIP_2 derivatives (i.e. IP_3) [50,51]. These observations suggest that PIP_2 hydrolysis might create a more conducive environment for cytoskeleton disassembly. Also, the membrane anchoring protein of actin filaments,

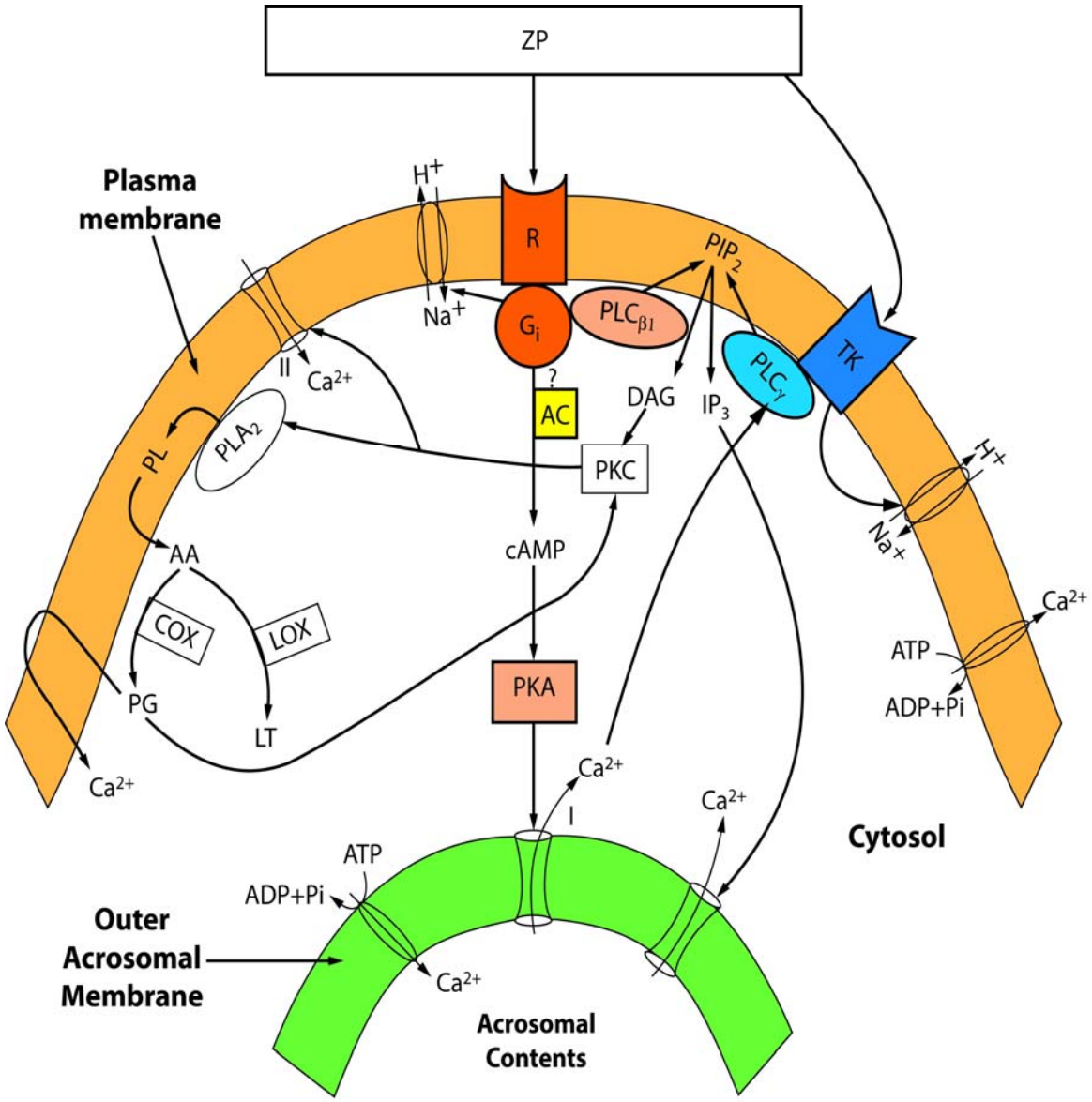

Figure 2. Interactions intrinsic to the acrosome reaction.

α-actinin, is associated with fatty acids within membranes including DAG. This could be another potential link to F-actin disassembly and the completion of the AR [48,52]. The activation of multiple metabolic pathways to ensure cytoskeleton disassembly and completion of the AR could potentially explain

why some AR inducers are not able to induce a complete AR (discussed in section below).

Some studies have suggested that it is not the acrosomal enzymes that permit ZP penetration, but mechanical forces (hypermotility and lateral head displacement) of the eutherian sperm cell that allows entry into the perivitelline space [53,54]. Evidence for non-lytic ZP access is based on the exclusion of single proteolytic enzymes, thought to be key zona lysins contained within the acrosome [55,56,57]. Even though Adham et al. [57] reported that sperm from a strain of mice deficient in the acrosomal enzyme acrosin were able to penetrate the ZP and fertilize the ovum, they did display delayed fertilization. Additionally, they demonstrated that in vitro fertilization with equally mixed sperm (sperm with and without the acrosin protein) resulted in only acrosin positive sperm cells successfully fertilizing oocytes. With over twenty known powerful hydrolyzing enzymes of acrosomal origin (Table 1), many believe that it is not the work of one, but potentially the combination of multiple enzymes that induce ZP dissolution [58,59]. Despite conflicting theories of ZP penetration, the consensus is that the AR is necessary to complete the fertilization process. Release of lytic enzymes from acrosomal stores assists in ZP penetration and exposes binding domains on the IAM. These findings support the acrosome reaction as a prerequisite for successful fertilization.

Table 1. Enzymes reported to be of acrosomal origin.

Proacrosin	Galactosidase
Acrosin	L-Fucosidase
Hyaluronidase	Metalloendoprotease
Acid proteinase	N-Acetylexcosaminidase
Arylamidase	N-Acetylglucosaminidase
Arylsulfatase	Neuraminidase
Calpain	Ornithin decarboxylase
Caproyl Esterase	Peptidyl Peptidase
Cathepsin D	Phosphatase
Cathepsin L	Phospholipase A
Collagenase	Phospholipase C
Esterase	

[20,46,60]

Acrosome Reaction Induction

Mammalian sperm that have completed capacitation are able to undergo the acrosome reaction in response to a number of physiological and pharmacological stimuli. Pharmacological inducers include cAMP analogues [60], phorbol myristate ester (PMA) and other PKC activators [61], platelet-activating factors [62], heparin [63,64], and calcium ionophore A23187 [13,18,40,64,65,66,67,68]. Physiological inducers like zona pellucida [69,70,71,72], progesterone [11,32,33,34,35,73], bicarbonate [73], or follicular fluid [74] have been used in studies to activate the acrosome reaction.

In vivo the AR can be initiated by sperm-zona binding in mammals, including stallions [65,71], boars [70], rams [75], and bulls [69,72]. Progesterone molecules are present in the environment of the ovulated oocyte; therefore, it is believed that progesterone might contribute to acrosomal exocytosis in an

additive or synergistic manner with ZP binding proteins, resulting in fertilization [34,76]. Under *in vivo* conditions, the acrosome reaction will follow ZP binding only if the spermatozoon has previously undergone capacitative maturation. Freshly ejaculated sperm of some mammalian species, including stallions [11,23,33,34], dogs [32], and bulls [33,34,35] will not bind with progesterone. After capacitation, both ZP and progesterone receptors are exposed on the sperm's plasma membrane enabling zona pellucida binding and AR induction. Even after capacitation progesterone treatment will only initiate the acrosome reaction and does not completely release acrosomal contents and/or superficial membranes from the sperm [18,73]. Other physiological stimulants such as bicarbonate [73] and follicular fluid [46] have also been shown to initiate the AR but not to stimulate enough of a response to expose the IAM. Similarly, pharmacologic acrosomal inducers such as platelet-activating factors [62], cAMP analogues, and PKC activators generate only partial reactions [46]. The purpose of the AR is to reveal oolema binding sites and penetrate the egg vestments. Inducers that do not stimulate a complete AR would not be able to reach or bind to the oolema, preventing fertilization.

It is also possible that some AR inducers are not able to generate sufficient calcium concentrations to sustain the AR because calcium is considered an essential component for the AR *in vitro* and *in vivo* [20,77]. The relationship of ionic calcium to induction of the AR requires time-course elevation of cytoplasmic Ca^{2+} concentrations. The AR requires a continual

increase of cytosolic calcium concentration as the signal transduction cascade progresses [44,46]. At least four enzymes require calcium at varying concentrations for operation : (1) PLC at submicromolar Ca^{2+} concentrations [49]; (2) PKC at micromolar Ca^{2+} concentrations [78]; (3) PLA at micromolar Ca^{2+} concentrations [79]; (4) actin severing proteins at supramicromolar Ca^{2+} concentrations [49,80].

Ionophores are compounds that form lipid-soluble complexes with polar cations to increase membrane permeability [81]. Diffusion rates of ionophore complexes across biological lipid bilayers are so favorable that rates can reach thousands per second [82]. Calcium ionophore A23187 generates a rapid influx of calcium to produce high intracellular calcium levels that triggers a non-regulated exocytosis of acrosomal contents [20,46]. This ionophore is capable of supplying Ca^{2+} dependent reactions with Ca^{2+} from external stores in exchange for H^+ without disturbing the pre-existing sodium and potassium balances at the surface [81]. However, acrosome reaction induction with A23187 differs significantly from the physiological sequence. The A23187 stimulates a rapid influx of calcium to trigger the acrosome reaction, and excludes the sequence of molecular events that naturally occurs during capacitation [20,46]. Studies indicate that A23187 efficiently induces a complete, non-physiologic acrosome reaction in sperm. Cummins et al. [83] and Tesarik [84] recommend the use of an ionophore challenge as a functional test to assist in diagnosis of infertility due to A23187's high correlation with fertilizing

capabilities of sperm. Zhang et al. [85] applied anti-acrosome monoclonal antibody HS 18-6 to horse semen and highly correlated a stallion's response to A23187 with their ability to penetrate and fertilize zona pellucida intact horse eggs. Calcium ionophore A23187 has been shown to stimulate the AR in multiple mammals [18,40,68,86,87].

Acrosomal Probes

Many techniques have been developed to evaluate acrosomal status of sperm. The size of the acrosome is species specific, making direct visualization of the acrosome limited to species with large acrosomes (e.g. guinea pig and hamster). Therefore, cytochemical techniques for species with small acrosomes (e.g. human and stallion) have been developed to assist with acrosomal assessment [65,88]. A triple stain technique employing Bismark Brown, Rose Bengal, and the supravital stain, trypan blue, has been found to be useful in mice [89], humans [88], stallions [14], goats [90], and elephants [91]. Other common methods are the labeling of the sperm plasma membrane with chlortetracycline (CTC) [14,92,93] and acrosomal constituents with specific monoclonal antibodies [94,95,96,97] or with fluorescent lectins [16,98]**.**

Chlortetracycline (CTC) fluorescence is emitted when CTC forms a complex with plasma membrane-bound calcium, emitting an enhanced fluorescence due to calcium influx associated with capacitation and the acrosome reaction [27]. Capacitative and acrosomal activity has been

monitored with CTC in multiple species; including mice [99, 100], humans [101], and stallions [14]. However, CTC staining techniques give rise to assorted staining patterns and introduce subjectivity when determining capacitative and acrosomal status [27,68].

Indirect immunofluorescence staining with monoclonal antibodies is highly suited to detect subcellular domains, but the technique is expensive and the reagents are not readily available commercially. Another drawback is that some selected antibodies target the IAM [18,102] and therefore only identify cells that have completely acrosome reacted.

There are a variety of lectins commonly used that bind to the glycoconjugates of acrosomal membranes as well as the acrosomal matrix. Peanut agglutinin (PNA) binds exclusively to β-D-galactose residues localized on the OAM of sperm [27]. This indicates PNA is useful for detecting initial acrosomal status (i.e. acrosome intact) but not completion since the PNA lectin would be lost with the OAM during the acrosome reaction. Pisum sativum agglutinin (PSA) lectin is specific for α-mannose moieties within the acrosomal matrix and is the probe of choice for acrosomal exocytosis detection [8,16,17,18,19]. Sperm containing intact acrosomes prevent PSA from coming in contact with the acrosomal contents to which it binds [103] and therefore the sperm membrane must be permeabilized if the matrix is to be labeled. Permeabilization of the sperm membranes produces an initial fluorescence staining of the acrosomal cap, followed by a decrease in fluorescence as the

acrosomal contents are released. A drawback of using lectins for acrosomal identification in the vicinity of oocytes is that they will also bind to glycoconjugates of oocyte origin and potentially produce false positives or obscure visualization of sperm [12].

Pisum sativum agglutinin has been selected for its ability to identify both partial and complete acrosome reactions [16,17,27]. Lectins can easily be conjugated to several different fluorescent compounds. Fluorescein isothiocyanate (FITC) is one of the most commonly used green fluorescent labels [104] and is routinely conjugated with PSA for fluorescence studies. Fluorescein isothiocyanate binds covalently with amino acids, producing a stable attachment that can withstand multiple washings [105]. It has been reported that FITC-PSA staining effectively monitors the exocytosis of acrosomal contents and the corresponding decrease in fluorescence intensity [11,15].

Paraformaldehyde is the fixative of choice for preserving the acrosomal status of the sperm cells. Paraformaldehyde maintains morphological integrity of acrosomal and plasma membranes of human sperm, allowing accurate staining of the membranes after fixation of the cells [106]. Using flow cytometry, Miyazaki et al. [107] generated regions to quantify the decrease in green fluorescence emitted from the FITC-PSA of acrosome reacting sperm samples of men. We fashioned similar regions to establish equine sperm standards for fluorescence intensities, rate of response to A23187, and percent reaction of sperm from fertile stallions utilizing flow cytometry.

Flow Cytometry

Several methods are used to assess the status of the acrosome, including transmission electron microscopy [11,13,17,108],and both light and fluorescence microscopy following cytochemical labeling of acrosomal membranes and/or acrosomal contents [8,19,65,68,73,87,88 109]. Transmission electron microscopy (TEM) is considered to be the gold standard for evaluation of the acrosome because of the direct visualization of cellular ultrastructure. Limitations of TEM include cost and time required to perform the tests. All microscopic methods (TEM, light microscopy, or fluorescent microscopy) are limited to evaluation of a small number of sperm (50-200). Flow cytometry provides a rapid, objective evaluation of large numbers (thousands) of sperm. Researchers find this technique to be an objective, efficient, and accurate procedure to estimate sperm acrosomal status of men [107,110], bulls [111], dogs [112], mice [113], and stallions [37].

Miyazaki et al. [1990] showed a high correlation ($r = 0.98$) between microscopy and flow cytometry when evaluating acrosome intact vs. reacted spermatozoa of human samples. Flow cytometric techniques are more objective and evaluate large numbers of sperm in a short period of time (seconds) [114]. Time course studies can also be performed where an immediate short-term acrosomal response can be measured following treatment. Additionally, flow cytometry has the ability to detect minute fluorescent changes in specific sperm

compartments that are not detected by the human eye even when utilizing microscopy.

Flow cytometers are manufactured by only a few companies but a variety of instruments are available for purchase. Various flow cytometers utilize different light sources to analyze and excite samples, with some containing up to 4 lasers. The FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems; San Jose, CA) uses an air-cooled, blue-green argon laser that emits light at 488nm, within the range of FITC excitation (Figure 3).

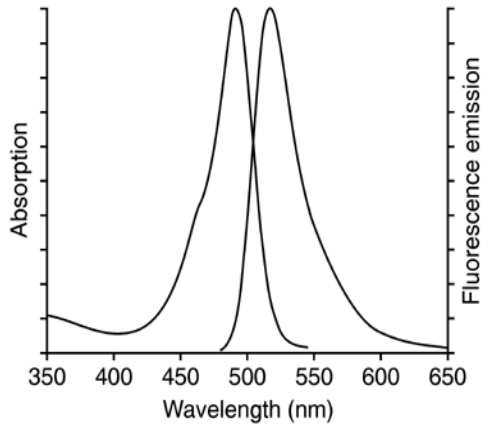

Figure 3. FITC absorption/emission spectrum by Molecular Probes.

When using flow cytometric methods, the user can gate out debris, differentiating it from sperm cells to ensure the purity of the analyzed data. Some flow cytometers can analyze up to 40,000 cells per second, the FACScan has a maximum analysis rate of 10,000 cells per second, to enable an efficient

and objective method of monitoring the changes of sperm's acrosomal status [105].

MATERIALS AND METHODS

Equipment, Chemicals, and Reagents

E-Z Mixin™ – Cool-Store/Transport (CST) skim milk -glucose equine semen extender, nylon micro-mesh filters, and the densimeter were supplied by Animal Reproduction Systems (Chino, CA). Equitainer™ transport containers were from Hamilton-Thorne Research (Danvers, MA). EquiPure™ Gradient was obtained from Nidacon International AB (Gothenburg, Sweden). Dulbecco's phosphate-buffered saline (DPBS) (Cat #14040-117) and fetal bovine serum (Cat #10082-139) were purchased from Gibco (Grand Island, NY). Calcium ionophore A23187 (Cat #100105) was purchased from Calbiochem (San Diego, CA). Sigma Chemical Company (St. Louis, MO) was the supplier for all other chemicals and reagents.

Animals and Semen

Semen for all experiments was obtained using a Missouri-model artificial vagina (AV), with the aid of either an ovarectomized mare or a mounting phantom as a mounting source. Ejaculates were collected from each stallion using an AV fitted with a nylon micro mesh filter to remove gel and debris. Semen was then transported to an adjacent laboratory and placed in a 37ºC incubator for no more than 5 minutes prior to beginning the experiments. Sperm concentration was determined photometrically (densimeter), then extended to $25x10^6$ sperm/mL with E-Z Mixin™ CST semen extender.

Calcium Ionophore A23187 Preparation

Stock solutions of 10mM A23187 in dimethylfulfoxide (DMSO) were prepared by adding 1mg A23187 to 190µL DMSO. The stock solution was stored at -20ºC until added to sperm suspensions, at a final A23187 concentration of 10µM.

Fluorescencein Isothiocyanate (FITC)-Pisum Stivum Agglutinin(PSA) Preparation

Fluorescencein isothiocyanate-pisum stivum agglutinin (FITC-PSA) (2mg) from Sigma (Catalog #L-0770) was utilized. Stock solutions were prepared by adding 8mL of DPBS to create a 0.25 mg/mL solution. The stock solution was separated into 30µL vials and stored at -20ºC until needed. The 30µL vials of FITC-PSA were further diluted with 120µL of DPBS to create a 0.05 mg/mL solution to be added to the sperm suspensions.

Paraformaldehyde Preparation

A vacuum hood was utilized as a safety precaution for preparation of paraformaldehyde solution. The paraformaldehyde powder (2g) was dissolved in 50mL sterile water and heated to approximately 50ºC, until the mixture went into solution. 5N NaOH was added drop wise until the solution became clear. The paraformaldehyde solution diluted 1:1 with 2X Dulbecco's phosphate-buffered saline (DPBS) to obtain a 2% (w/v) paraformaldehyde solution. The pH

of the solution was adjusted to 7.2 - 7.4, by drop-wise addition of 1N HCl. The paraformaldehyde solution was prepared weekly and stored at 4ºC until used. We saw no changes in staining pattern over a one week period and had no reason to suspect that the paraformaldehyde needed to be made daily.

Fetal Bovine Serum Preparation

Twenty percent (v/v) fetal bovine serum (FBS) was prepared by dissolving 200µL FBS in 800µL DPBS. The FBS was used to inhibit free aldehydes from interfering with lectin binding [103].

Flow Cytometry

Data were recorded on cellular events using a FACScan Analyzer flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). The FACScan is equipped with an air-cooled argon laser that emits light at 488nm. Green fluorescence from FITC-PSA was collected through a 530/30-nm band-pass filter with logarithmic amplification. Both forward and side light scatter were acquired. Voltage settings used were: green fluorescence (FL1) = 798; forward scatter = E00; and side scatter = 240. Five thousand sperm cells were acquired using Cell Quest™ Software (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Sperm were gated based on their forward and side scatter properties (Figure 4). Gating allowed the evaluation of only cells that met the size specifications of stallion sperm, limiting the analysis of debris [105]. Data were

stored in list mode format and analyzed using the flow cytometry analysis software Win List™ (Verity Software House, Inc., Topsham, ME).

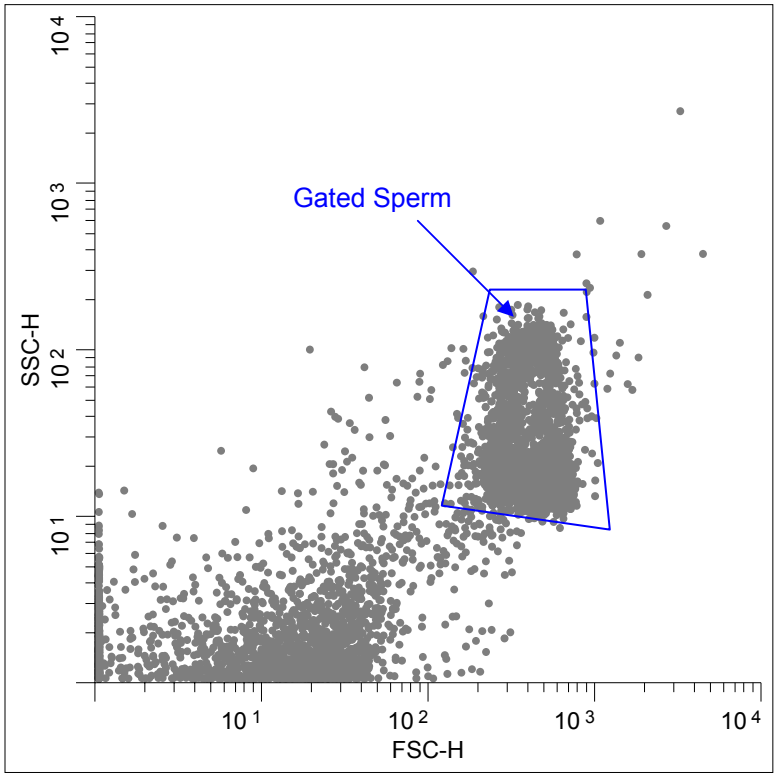

Figure 4. Forward and side scatter of stallion semen.

For initial flow cytometric studies, Equipure™ density gradient separation was applied to ejaculates obtained from 3 fertile stallions to optimize spermatozoal quality in an effort to determine the acrosome-intact region for scattergrams for all experiments (Procedure in Appendix A). A 95% confidence interval was used to create an acrosome intact region with a width of 80 channels [Region 3 (R3)]. Region 3 was centered on the mean of the initial control (Control 0hr) sample for

each stallion, and remained constant throughout the incubation periods. Total mean green fluorescence intensity [Region 1 (R1)] and the percentage of acrosome intact sperm were recorded for all samples. Figure 5 is a representative of the green fluorescence intensity histograms of fertile stallion sperm after 0, 1, 2, and 3 hours of A23187 exposure.

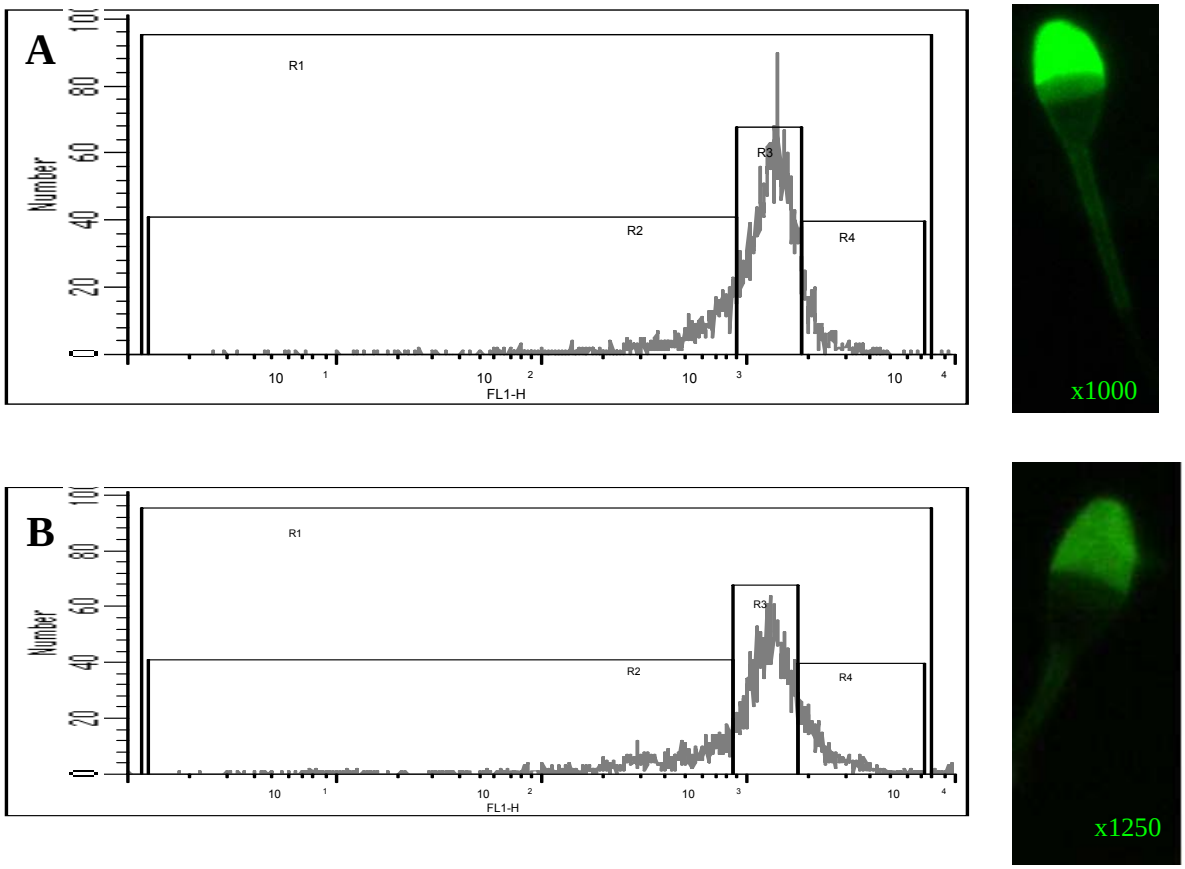

Figure 5. Green fluorescence intensity histogram of fertile stallion semen. R1) Total mean fluorescence of the sample. R3) Region of acrosome intact spermatozoa. A23187 treatment at A) 0h and a representative spermatozoon shown with fluorescence microscopy; B) 1h; C) 2h and representative spermatozoon; D) 3h and representative spermatozoon.

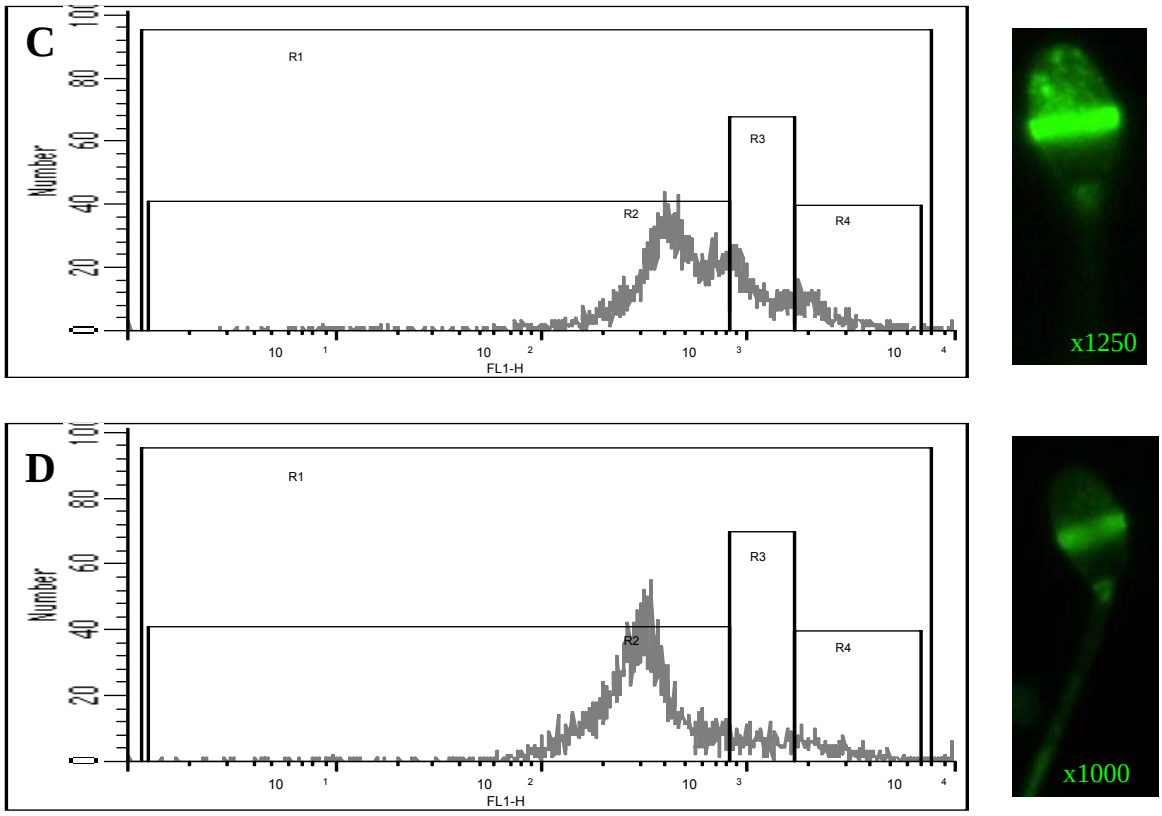

Figure 5. (Continued)

Assay Precision

To determine the repeatability of the assay, percent acrosome intact and total mean green fluorescence were analyzed from one ejaculate of a fertile stallion. Intra-assay (between tubes) variability was measured for 6 replicates each of the control and A23187-treated aliquots. Intra-assay (within tube) variability was measured on 6 replicates from a single tube of each treatment. Inter- and intra-assay variability was determined by coefficient of variation (CV). The CV provides a measure of variation relative to the mean.

Statistical Analysis

To model acrosomal status, a mixed regression model was designed in which error terms were modeled for stallion, ejaculate within stallion, and individual treatment. This model evaluated the effects of A23187 on the incidence of acrosome-intact sperm and total mean green fluorescence of post-fixation stored samples, cooled semen, and within stallion categories (fertile or subfertile).

Incubation, Permeabilization, and Fluorescence Staining

Freshly-ejaculated semen was extended to $25x10^6$ sperm/mL with EZ Mixin-CST equine semen extender. The extended semen sample was divided into 1-mL aliquots to create control (no A23187) and perturbed (10µm A23187) treatment groups. Three replicates of each treatment per incubation period (0, 1, 2, and 3 hours) were prepared. Pre-fixation incubation treatment groups are summarized in Table 2.

Table 2. Treatment design for all experiments.

Pre-fixation Incubation @ 37ºC			
0hr	**1hr**	**2hr**	**3hr**
A23187	A23187	A23187	A23187
Control (No A23187)	Control	Control	Control

Extended semen in the two treatment groups was incubated at 37ºC for 0, 1, 2, and 3 hours. Following specified incubation periods (0, 1, 2, or 3 hr) samples were centrifuged (320Xg for 4 minutes) then fixed with 1mL 2% paraformaldehyde for 10 minutes at room temperature. Due to processing time, incubation period zero (0hr) were not fixed for approximately 5 minutes after ejaculate was extended. After paraformaldehyde fixation, samples were washed once, then resuspended in 1mL of DPBS and stored at 4ºC for 0, 24, and/or 72 hours. Following post-fixation storage of sperm, acrosomal membranes were permeabilized with 95% ethanol (v/v) (-20°C) for 10 minutes, then washed with DPBS (320Xg for 4 minutes). Samples were then resuspended in 20% (v/v) fetal bovine serum and labeled with FITC-PSA for 10 minutes at room temperature [19]. The samples were then washed twice. After the second wash, the supernatant was removed and 10µL of the sample was suspended in 1mL of DPBS and analyzed by flow cytometry.

Experimental Procedures

Experiments I-III, semen was treated as presented in Table 2.

Experiment I – Effect of pre-fixation incubation at 37°C and post-fixation storage at 4°C on spermatozoal acrosome status and function

Three ejaculates from three fertile stallions were used for this experiment. Samples were processed as per 'Incubation, Permeabilization, and Fluorescence Staining'. However, following paraformaldehyde fixation samples

were washed with DPBS once (320Xg for 4 minutes), then resuspended in 1 ml of DPBS and stored at 4ºC in a digital refrigerator for 0, 24, and 72 hours, representing post-fixation storage. After the appropriate post-fixation storage, the protocol was completed.

Experiment II – Effect of Equitainer™ storage on spermatozoal acrosome status and function

Two ejaculates from three fertile stallions were used for this experiment. Sixty milliliter aliquots of fresh-extended semen ($25x10^6$sperm/mL) were packaged and placed in an Equitainer™ semen transport containers then removed after 24 hours of storage (i.e. simulating semen transport at ~7°C) to be processed as described above in 'Incubation, Permeabilization, and Fluorescence Staining'. These samples followed the 24-hour post-fixation storage. Permeabilization and fluorescence staining were then performed, and results were compared with the 24-hour post-fixation storage group in Experiment I which was not subjected to fresh storage.

Experiment III – Comparison of acrosomal status and function between fertile and subfertile stallions

Semen was collected from 15 fertile and 4 subfertile stallions and processed as described in above in the 'Incubation, Permeabilization, and Fluorescence Staining' in the previous section following a72-hour post-fixation

storage period at 4°C. Breeding records were obtained from owners. Initial spermatozoal motility and morphology measures were determined by a single operator. Motility values were determined subjectively using a microscope equipped with phase-contrast optics and a heated stage, or with a computer assisted sperm motility analyzer (IVOS Version 10; Hamilton Thorne Biosciences, Beverly, MA). Morphology data were collected by fixing samples in buffered-formalin solution, followed by evaluation with differential interference microscopy (Olympus BX-60, Olympus America Inc., Melville) at 1250 x magnification. Spermatozoal morphologic characteristics were classified according to the guidelines of the Society for Theriogenology [115].

RESULTS

Assay Precision

A fertile stallion semen sample was utilized and processed as per 'Incubation, Permeabilization, and Fluorescence Staining' following the pre-fixation incubation time of 0hr only. Intra-assay (within tube) precision revealed that the flow cytometry enabled repeatable acquisition of fluorescence intensity of the samples, with a CV less than 1 (Table 3). Inter-assay (among tube) CV was lower for mean fluorescence (Control: 1.32; A23187: 1.26) than for the percentage of acrosome intact sperm (Control: 10; A23187: 5). Overall, the staining and flow-cytometric methods were repeatable (CV $\leq$ 10%).

Table 3. Intra-assay variability for total mean green fluorescence and percent acrosome intact sperm, as measured by flow cytometry.

Intra-assay Precision (within tube)				**Intra-assay Precision (among tubes)**			
Treatment	**Mean**	**SD**	**CV**	**Treatment**	**Mean**	**SD**	**CV**
Mean Fluorescence Units							
Control	752	2.40	0.32	Control	747	10	1.32
A23187	741	2.96	0.40	A23187	747	9	1.26
Acrosome Intact (%)							
Control	76	0.70	0.93	Control	66	7	10
A23187	74	0.30	0.41	A23187	75	3	5

Experiments

Experiment I – Effect of pre-fixation incubation at 37°C and post-fixation storage at 4°C on spermatozoal acrosome status and function

Control and A23187-treated samples of storage period zero (S0) were not different (P>0.4) in percentage of acrosome intact (AI) sperm throughout the first

hour of incubation (Figure 6). After two hours of incubation ionophore treated samples exhibited a decrease (P<0.0001) in AI sperm, as compared to the control samples. Control samples decreased 20% after 3 hours of incubation, as compared to a decrease of 60% in the treated samples. For fresh semen

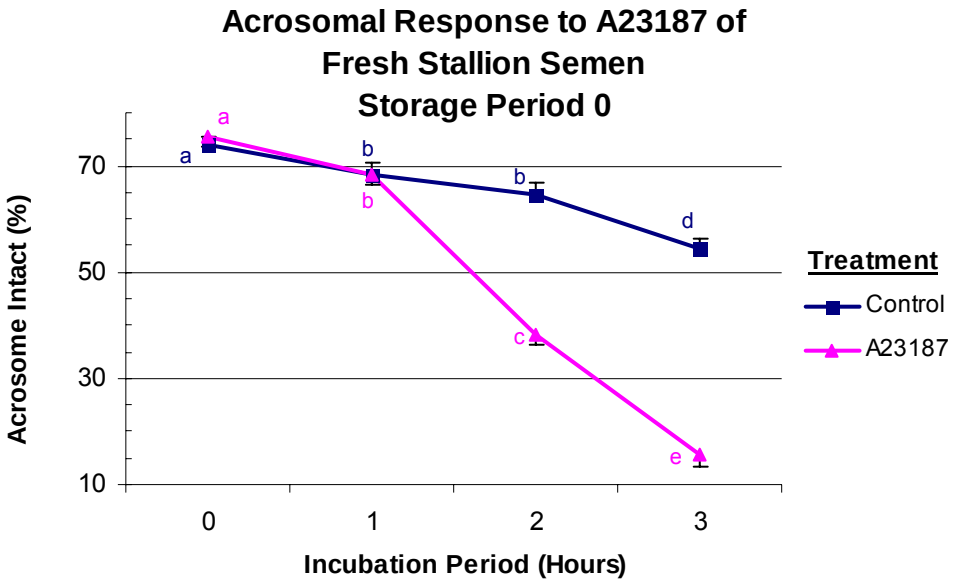

Figure 6. Mean percent acrosome intact (± S.E.M) sperm (n = 9 ejaculates from 3 stallions) from fertile stallions labeled with FITC-PSA. Different letters (a-e) indicate a significant difference (P<0.05) among data points.

(storage period 0), the mean green fluorescence intensity of control and treated samples declined throughout the incubation periods (Figure 7). As with the percentage of AI cells, no significant difference (P<0.05) in fluorescence intensity occurred between treatments until the second hour of incubation. Controls of the fresh samples lost a total of 36 fluorescence units by the third

hour of incubation, while A23187 treated samples had a fluorescence-intensity loss of over 98 units. The overall mean percentage of AI sperm for controls and

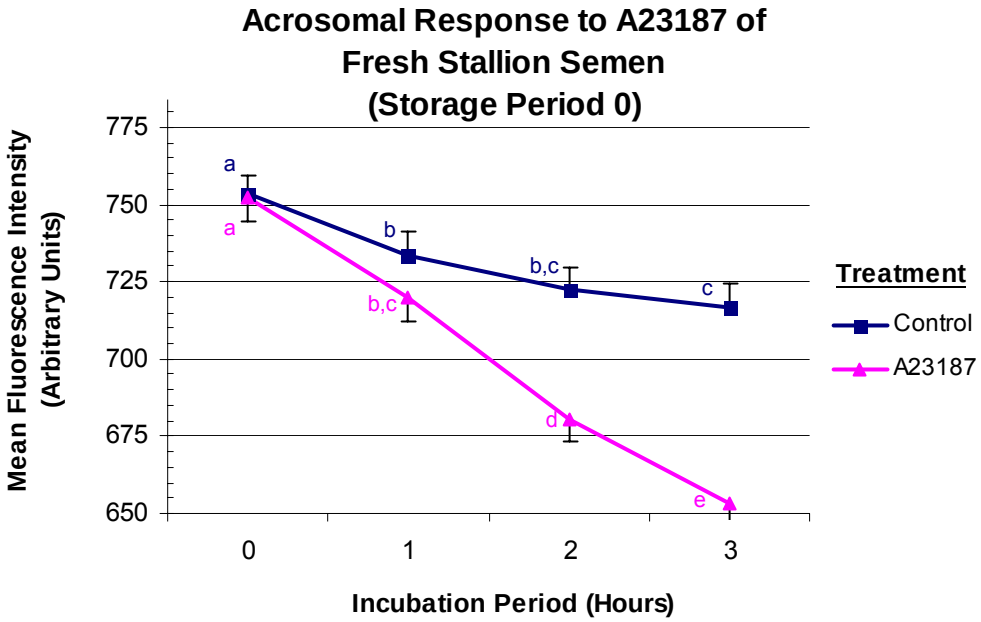

Figure 7. Mean fluorescence intensity (± S.E.M) sperm (n = 9 ejaculates from 3 stallions) from fertile stallions labeled with FITC-PSA. Different letters (a-e) indicate a significant difference (P<0.05) between data points.

treated samples at post-fixation storage period 0 (S0) was greater (P<0.05) than storage for 24 (S24) or 72 (S72) hours (57±1.14%, 54±1.14%, and 53±1.14%, respectively; Figure 8). Percentages of AI sperm did not differ (P=0.34) between Groups S24 and S72. For fresh semen (i.e. no 37^{o}C incubation), Group S0 had more (P<0.001) AI sperm than S24 and S72 groups (74±1.62%, 67±1.62%, 66±1.62%, respectively; Figure 9). Following 1 or 2 hours of pre-fixation incubation, the percentage of AI did not differ (P>0.1) among post-fixation

storage periods. After 3 hours of incubation, Group S0 (54±2.09%) contained fewer (P<0.05) AI sperm than Group S24 (60±2.09%).

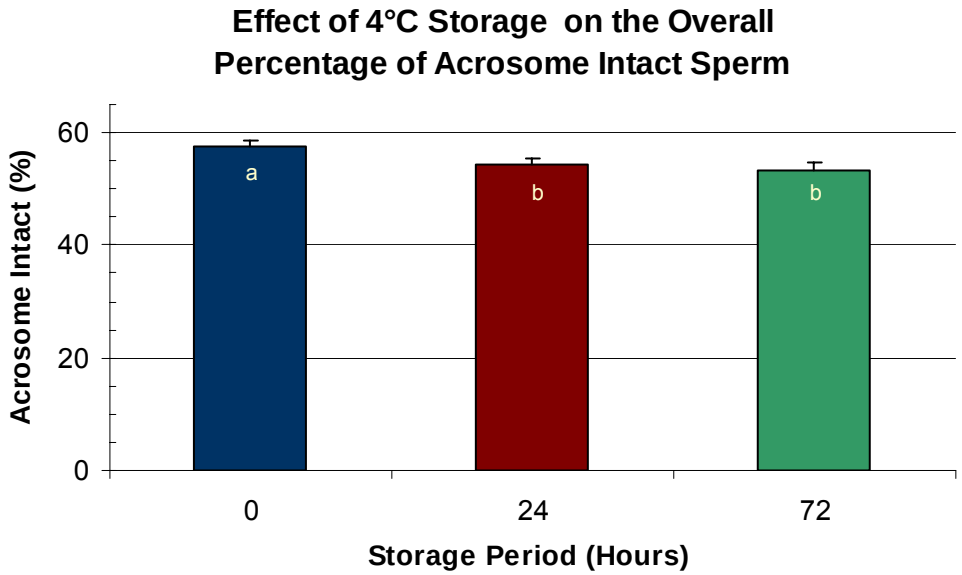

Figure 8. Overall (control and A23187 treated combined) mean percent acrosome intact (± S.E.M) sperm (n = 9 ejaculates from 3 fertile stallions) labeled with FITC-PSA after fixation, plus post-fixation storage at 4°C for 24 or 72 hours. Columns with different letters (a,b) are significantly different (P<0.05).

Similarly, A23187-treated samples for Group S0 (76±2.09%) at Incubation Period 0 contained significantly more (P<0.01) AI cells than Groups S24 (68%±2.09) or S72 (65%±2.09; Figure 10). However, there was no difference (P>0.2) in the percentage of AI sperm among storage periods following the first hour of incubation. After 2 hours of incubation, S24 and S72 for A23187-treated samples had fewer (P<0.05) AI sperm than Group S0. Samples processed immediately (S0) and stored (S24 and S72) samples exhibited similar (P>0.25) percentages (~14%) of AI sperm cells following three hours of ionophore incubation.

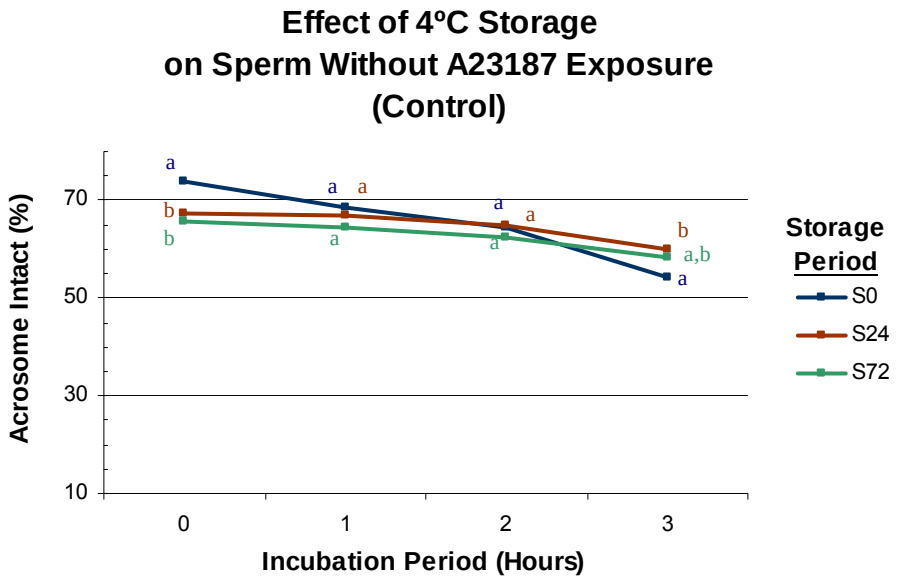

Figure 9. Mean percent acrosome intact (± S.E.M) of fertile stallion sperm (n = 9 ejaculates from 3 stallions) labeled with FITC-PSA after post-fixation storage at 4°C for 0, 24, and 72 hours (S0, S24, S72, respectively). Within an incubation period, different letters (a,b) are significantly different (P<0.05).

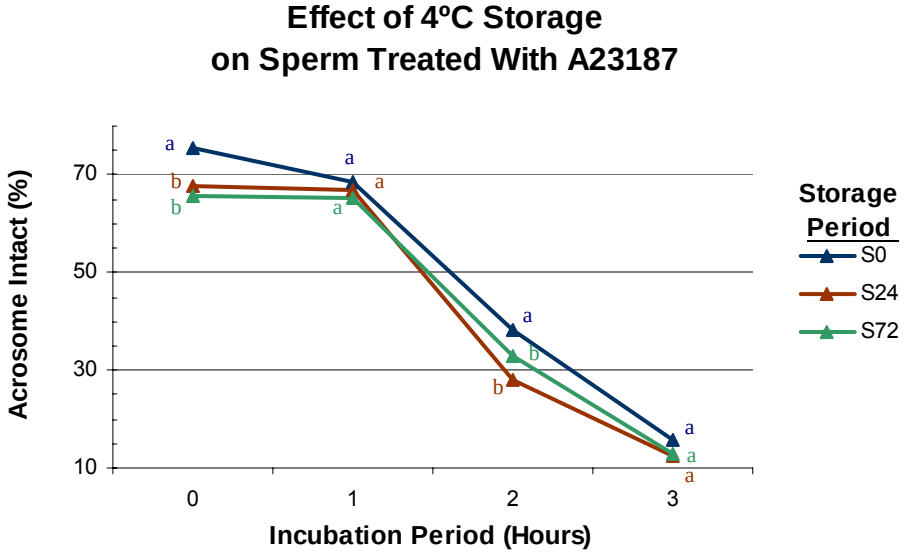

Figure 10. Mean percent acrosome intact (± S.E.M) of fertile stallion sperm (n = 9 ejaculates from 3 stallions) labeled with FITC-PSA after post-fixation storage at 4°C for 0, 24, and 72 hours (S0, S24, S72, respectively). Within an incubation period, different letters (a,b) are significantly different (P<0.05) .

The overall sperm fluorescence intensity was significantly different ($P<0.05$) among storage periods, with total fluorescence increasing progressively through 0, 24, and 72 hours of storage (717±5.36, 743±5.36, and 749±5.36 fluorescence units respectively; Figure 11). Mean fluorescence intensity for Group S0 was lower ($P<0.0001$) than both S24 and S72 groups (Figure 12) throughout the three hours of incubation. However, controls of all three storage periods produced a slow, steady decrease in fluorescence throughout the incubation periods, losing approximately 30 units of fluorescence by the third hour of incubation.

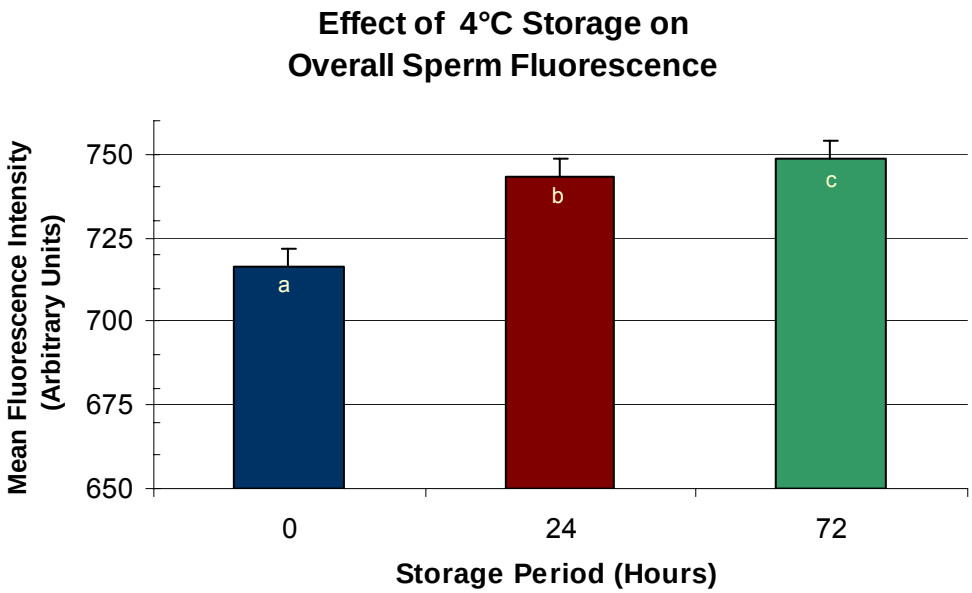

Figure 11. Overall (control and A23187 treated combined) mean fluorescence (± S.E.M) of fertile stallion sperm (n = 9 ejaculates from 3 stallions) labeled with FITC-PSA after fixation and storage for 0, 24 or 72 hours. Columns with different letters (a-c) are significantly different ($P<0.05$).

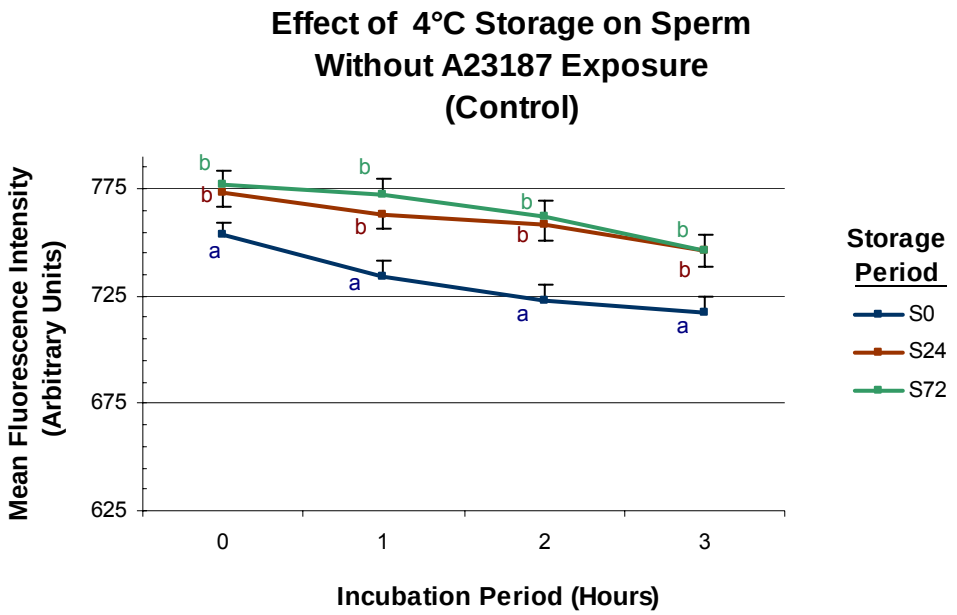

Figure 12. Mean fluorescence (± S.E.M) of sperm (n = 9 ejaculates from 3 stallions) after post-fixation storage at 4°C for 0, 24, and 72 hours (S0, S24, and S72, respectively). Within an incubation period, different letters (a,b) are significantly different (P<0.001).

Similarly, total green fluorescence for sperm treated with A23187 was less (P<0.0001) in Group S0, than in Groups S24 or S72 for all incubation periods (Figure 13). Ionophore treatment of fresh samples (S0) declined (P<0.001) in fluorescence intensity between each incubation period, whereas stored samples did not change (P>0.1) until after one hour of A23187 incubation. Ionophore treatment of both fresh and stored samples stimulated a 13% mean reduction in total sperm fluorescence by the third hour of incubation (i.e., unit losses of 99, 101, and 110 for S0, S24, and S72 respectively).

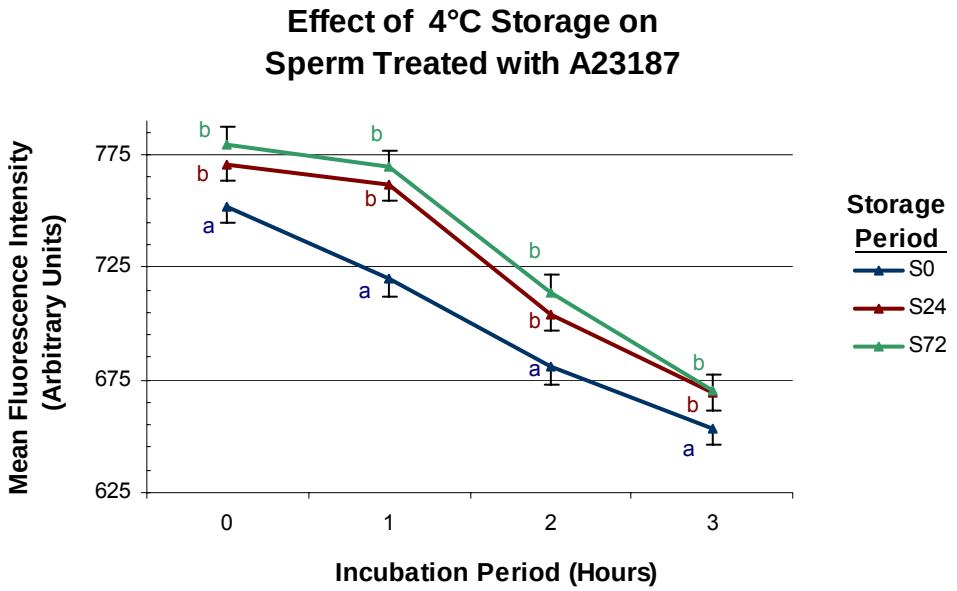

Figure 13. Mean fluorescence (± S.E.M) of sperm (n = 9 ejaculates from 3 stallions) after post-fixation storage at 4°C for 0, 24, or 72 hours (S0, S24, and S72, respectively). Incubation periods with different letters (a,b) are significantly different (P<0.05).

Experiment II - Effect of Equitainer™ storage on spermatozoal acrosome status and function

The overall (control and ionophore groups combined) percentage of AI sperm stored in the Equitainer™ was approximately 6% lower (P<0.0001) than the fresh samples (Figure 14). Initially, both the fresh and Equitainer™-stored samples produced similar (P>0.2) percentages (~ 62%) of AI sperm cells (Figure 15). After one hour of 37°C incubation, Equitainer™-stored samples began to deviate from the fresh samples. The fresh samples had significantly more (P<0.05) AI sperm than Equitainer™-stored samples throughout the remainder of 37°C incubation.

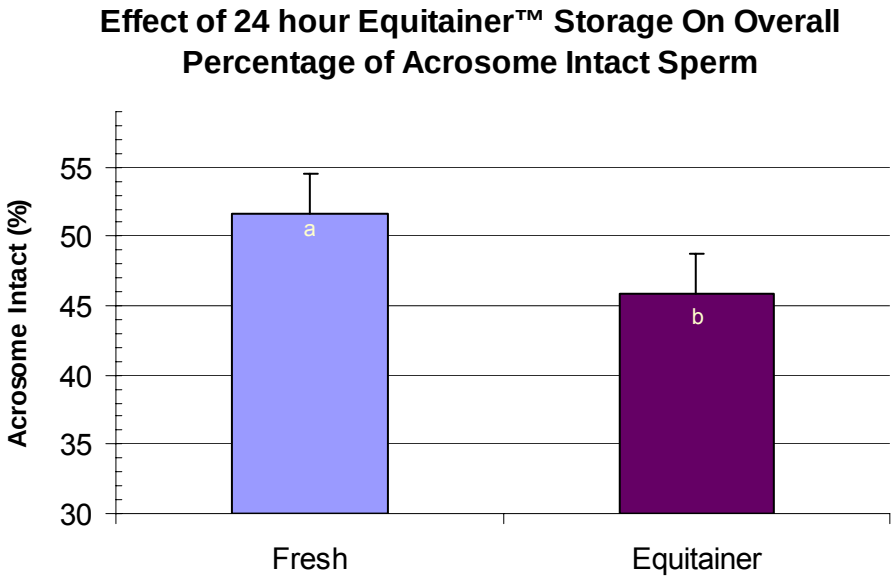

Figure 14. Overall (control and A23187 treated combined) mean percent acrosome intact sperm (n = 6 ejaculates from 3 stallions) of fresh and 24h Equitainer™ stored sperm. Different letters (a,b) indicate the significant difference (P<0.0001) between storage environments, where fresh samples contained 5.82% more AI sperm cells.

Controls groups showed no difference (P>0.1) in percentage of AI sperm between fresh and Equitainer™-stored samples except at the second hour of incubation, where fresh samples contained almost 10% more intact cells. Ionophore treatment of fresh sperm contained significantly more (P<0.05) AI cells than Equitainer™-stored semen after the first and third hours of incubation. Following three hours of ionophore exposure, fresh semen contained significantly more (P<0.01) AI sperm than Equitainer™-stored samples (20 vs. 10% respectively).

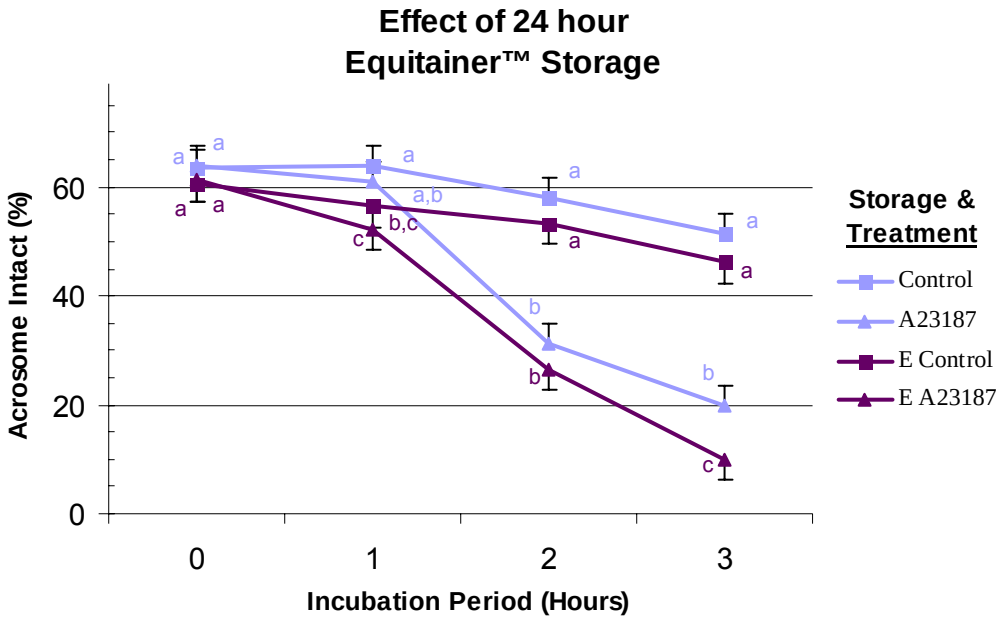

Figure 15. Mean percent acrosome intact sperm (n = 6 ejaculates from 3 fertile stallions) after 24h Equitainer™ (E) and without Equitainer™ storage. Different letters (a-c) indicate a significant difference (P<0.05) within an incubation period.

Overall (control and treated samples combined) mean fluorescence intensities of sperm were not different (P>0.21) between fresh (734±8.01) and Equitainer™-stored (730±8.08) samples (Figure 16). The fresh and stored samples exhibited a decrease of fluorescence intensity in the control and A23187 treated samples of 37 and 97 fluorescence units, respectively.

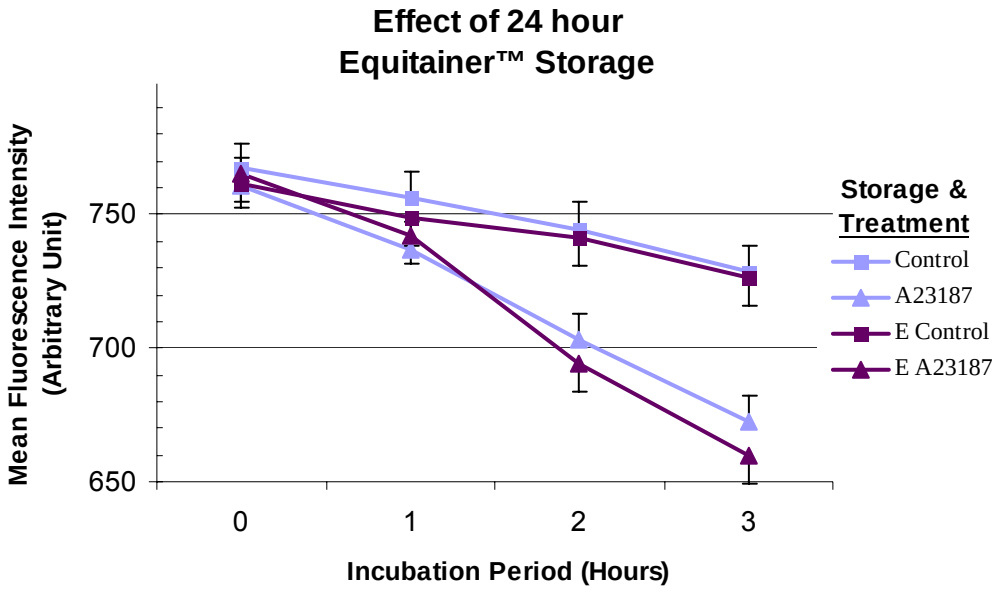

Figure 16. Mean fluorescence of sperm (n = 6 ejaculates from 3 fertile stallions) after 24h Equitainer™ (E) and without Equitainer™ storage. There is no significant difference (P>0.2) in fluorescence between fresh and Equitainer™ stored semen.

Experiment III – Comparison of acrosomal status and function between fertile and subfertile stallions

Values for spermatozoal motility and morphology from fertile and subfertile stallions are provided in Table 4. Breeding records of the fertile and subfertile stallions are provided in Table 5.

Table 4. Comparison of sperm characteristics between fertile and subfertile stallions.

Characteristic	Fertile Stallions Mean±sd (Range) n=16	Subfertile Stallions Mean±sd (Range) n=4
Volume (mL)	44±16 (15-71)	45±29 (20-87)
Concentration (x 10^6/mL)	205±85 (48-360)	160±31 (121-196)
Total Sperm Number (x 10^9)	8±4 (3-20)	8.38±7.81 (3.34-19.92)
Motility		
Extended Motility (%total)	80±12 (50-95)	74±10 (62-86)
Extended Motility (%progressive)	74±12 (45-90)	64±8 (53-73)
Velocity (0-4)	3.77±0.41 (3-4)	4.00
Morphology (%)		
Initial Acrosome Intact	59±9 (38-72)	60±12 (49.47-71.66)
Normal	58±22 (20-91)	66±16 (54-84)
Abnormal Acrosome	3±8 (0-27)	0.5±0.71 (0-1)
Abnormal Heads	10±8 (0-25)	11±10 (0-18)
Tailless Heads	1.57±2.11 (0-7)	1.33±1.15 (0-2)
Proximal Droplets	12±12 (0-46)	12±3 (10-15)
Distal Droplets	7.97±8.76 (0-32)	5±3 (2.0-7.0)
Abnormally-Shaped Midpieces	9±6 (1.0-22)	6±3 (3.0-9.0)
Bent Midpieces	1.72±2.45 (0-14)	3.67±4.04 (0-8)
Bent Tails	2.28±2.20 (0-6)	0.5±0.71 (0-1)
Coiled Tails	2.01±1.99 (0-6)	0
Premature Germ Cells	0.54±0.61 (0-2)	1.33±1.53 (0-3)
Other Abnormalities	0	0

Table 5. Comparison of fertility measures between fertile and subfertile stallions.

Characteristic	Fertile Stallions Mean±sd (Range) n=16	Subfertile Stallions Mean±sd (Range)
Breeding History		
Mares in Book	71±43 (4-133)	52±52 (5-108) [n=3]
% Maiden Mares Pregnant	88±11 (67-100)	82 [n=1]
% Foaling Mares Pregnant	88±8 (71-100)	49 [n=1]
% Barren Mares Pregnant	87±7.62 (74-100)	58 [n=1]
Maiden Mares Cycles/Pregnancy	1.60±0.43 (1-2.33)	1.67 [n=1]
Foaling Mares Cycles/Pregnancy	1.75±0.36 (1-2.2)	2.94 [n=1]
Barren Mares Cycles/Pregnancy	2.10±0.44 (1.7-3.09)	3.09 [n=1]
# Pregnant Mares	62±38 (4-121)	38±28 (18-58) [n=2]
% Pregnant Mares	89±7 (73-100)	29±24 (0-54) [n=4]
Cycles/Pregnancy	1.68±0.36 (1-2.39)	3±2 (2.83+) [n=4]

Sperm from control groups (no A23187) of fertile stallions did not change ($P>0.1$), maintaining approximately 57% of AI sperm, until incubation period 3 (51%±2.75). Control groups for subfertile stallions also exhibited no change ($P>0.4$) in the percentage of AI sperm throughout incubation periods 0, 1, 2, and 3 (58±5.00%, 57±5.28%, 57±5.28%, 60±5.28%, respectively). There was no difference ($P<0.05$) in the percentage of AI sperm between control groups of fertile and subfertile stallions throughout the incubation periods (Figure 17). Ionophore treatment of sperm from fertile stallions revealed a significant difference ($P<0.01$) in the percentage of AI cells among incubation periods. Ionophore treatment of sperm from subfertile stallions produced no significant

changes ($P>0.05$) in AI cells until the third hour of incubation. Three hours of ionophore exposure to sperm from fertile stallions stimulated a decrease of 35% in AI cells, as compared to a decrease of 11% in subfertile stallions.

Control groups from subfertile stallions exhibited no significant change in total fluorescence ($P>0.05$) among incubation periods, maintaining an approximate fluorescence intensity of 737±16.3 units. Control samples from fertile stallions maintained their fluorescence for incubation periods 0, 1, and 2 (746±8.18, 745±8.48, and 737±8.56, respectively), but the fluorescence intensity decreased ($P<0.01$) to 720±8.53 by the third hour of incubation. Ionophore treatment of sperm from fertile stallions produced a decrease ($P<0.001$) among incubation periods for fluorescence intensity. Subfertile sperm displayed no significant changes ($P>0.1$) in fluorescence after one hour of ionophore exposure. Ionophore treated sperm from subfertile stallions also displayed no significant difference ($P>0.1$) from their respective control groups or the control groups from fertile stallions throughout the incubation periods in mean fluorescence intensity (Figure 18). Similarly, the overall decrease in total sperm fluorescence was lower ($P<0.05$) in subfertile A23187 treated samples than for similarly treated samples from fertile stallion (38 vs. 75 units, respectively).

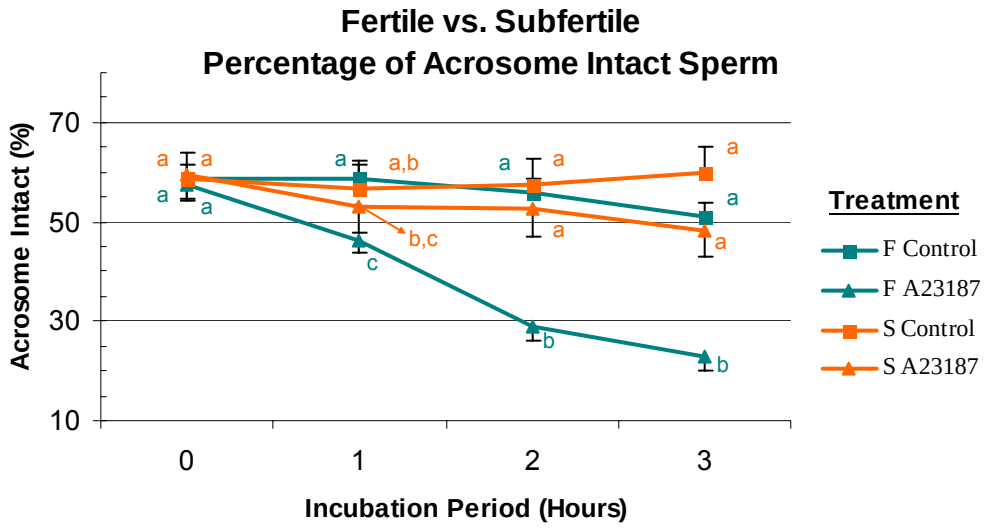

Figure 17. Mean percent acrosome intact (± S.E.M) of fertile (F) and subfertile (S) stallion sperm (n = 15 and n=4 respectively) after post-fixation storage at 4°C for 72 hours. Within an incubation period different letters (a,b) indicate a significant difference (P<0.05).

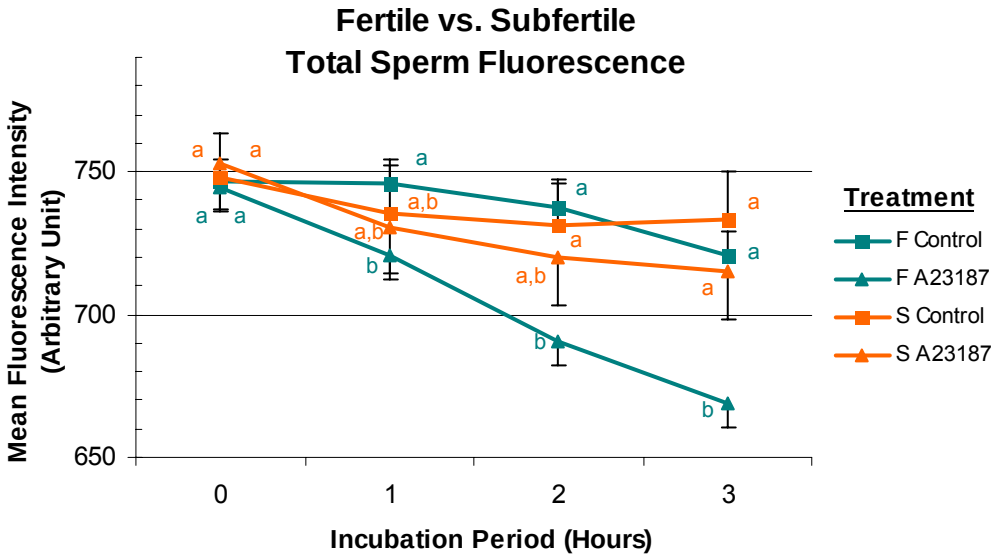

Figure 18. Mean fluorescence (± S.E.M) of fertile (F) and subfertile (S) stallion sperm (n = 15 and n=4 respectively) after post-fixation storage at 4°C for 72 hours. Within an incubation period different letters (a,b) indicate a significant difference (P<0.05).

CONCLUSION

This study utilized a reproducible method of staining acrosomes with FITC-PSA to determine acrosomal status and reactivity in stallion sperm. Fresh (S0) semen from fertile stallions at incubation period 0 contained approximately 75% acrosome intact (AI) sperm. These findings correspond to Farlin et al. [1992] who reported that 16% of fresh stallion sperm had damaged acrosomes. Fresh semen from fertile stallions showed an overall decrease in total fluorescence intensity, relating to the overall decrease in percentage of AI sperm in both controls and ionophore-treated samples. The decrease in AI cells in control samples suggests that more than 5% of the cells acrosome react or are damaged within 1 hour of incubation at 37ºC. More cells acrosome reacted between the 2nd and 3rd hour of incubation. These findings could be related to the heterogeneous population of sperm within an ejaculate and their capacitation and acrosome reaction rates, as has been described for sperm from hamsters [116] and humans [117]. Asynchronous capacitation would provide a higher probability of acrosomal reactivity of sperm when they are in the vicinity of the fertilization site [20]. Nagae and Yanagimachi [118] suggested that there could be an enhancement in PSA-staining at the initial stages of the AR due to acrosomal matrix swelling which increased the staining intensity. Using flow cytometry, Nikolaeva et al. [110] first reported an increase in PSA-staining intensity of human sperm at the onset of the acrosome reaction (i.e. early A23187 exposure). It is thought that the acrosomal matrix swells and the

volume between the OAM and the IAM increases during the initial stages of the AR. This increase in space allows for more glycoproteins to become more readily available to lectin binding, thus generating higher fluorescence intensity. Nonetheless, our studies did not reveal a significant difference (P=0.85) in initial fluorescence intensities between the ionophore treated samples and the respective controls.

Paraformaldehyde fixation of stallion sperm and the subsequent storage at 4ºC for 24 and 72 hours resulted in fewer initial AI cells than samples processed immediately (S0). These results suggest that there is some degradation (>5%) of acrosomal integrity associated with post-fixation storage. The results of this study also suggest that post-fixation storage increased spermatozoal sensitivity to ionophore exposure, i.e. the rate of response to A23187 was faster following post-fixation storage than in the respective S0 samples. Post-fixation storage could cause a capacitation-like membrane destabilization in some sperm to enable the release of acrosomal membranes and contents, which would mimic the physiological acrosome reaction. However, the results of this study suggest that post-fixation storage of stallion semen for up to three days is still representative of the acrosomal responsiveness of the original sample.

Mean fluorescence intensity of both storage periods (24 and 72 hours) were higher (P<0.0001) in both control and ionophore treatments of S0 samples. Also, overall mean fluorescence increased as the post-fixation storage was

prolonged. The increased fluorescence could be attributed to potential osmotic swelling of the sperm during storage and conveyed as an increase in fluorescence due to the increased availability of lectin binding sites. These intrinsic changes in fluorescence are minor; yet these alterations could be detected when utilizing flow cytometry for measurements.

The extensive use of cooled-shipped semen justifies the evaluation of sperm after cooled storage. Cooled stallion semen frequently suffers a decline in spermatozoal fertility that can vary among individuals. Brinsko et al. [2] showed that stallion semen extended in skim milk and packaged in an Equitainer™ for 24 hours caused over 10% of the cells to loose their progressive motility. Malgren [119] reported that the same conditions generated more than a 10% decrease in intact plasma membranes of stallion sperm. The decreased fertility of transported semen can also be attributed to acrosomal integrity. The results of the present study concluded that stallion semen extended in a skim milk extender and stored for 24 hours in an Equitainer™ transport container yielded almost 6% fewer AI sperm than the respective fresh samples. Cooled storage of extended stallion semen also stimulated a higher responsiveness to A23187. Plasma membrane alterations and/or damage during storage could stimulate membrane destabilization initiating capacitative changes that would shorten the life of the sperm and prematurely activate cellular metabolism. The development of a more suitable extender than the commercialized skim milk transport extender might induce a lesser degree of acrosomal disruption, since

skim milk-based extenders have been shown accelerate capacitative and acrosomal responses in stallion semen [19]. Despite the decrease in the percentage of AI cells, cooled-storage in an Equitainer™ created no difference ($P>0.2$) in mean fluorescence intensity compared to fresh semen in the present study. Unlike the post-fixation storage samples, cooled storage of fresh sperm did not alter the fluorescence properties of the sample. It is possible that live sperm are able to retain cytoplasmic constituents and maintain osmotic balances to preserve the fluorescence properties of the fresh sample. Nonetheless, Equitainer™ storage for 24 hours generated a sample with decreased acrosomal integrity, as compared to the original sample.

It has been demonstrated that subfertile stallions with below-average motility and/or morphology can also produce sperm with lower acrosomal reactivity to physiologic inducers such as progesterone and solublized ZP [11,12]. However, routine breeding soundness examinations do not always reveal the primary etiology for reduced fertility in stallions. Although normal motility and morphology characteristics exist, some stallions are not producing adequate pregnancy rates. Varner et al. [13] analyzed stallions with unexplained fertility and verified with TEM that the samples from subfertile stallions contained less acrosome-reacted sperm than fertile stallions, either spontaneously, or when exposed to A23187. In this study, flow cytometry enabled the quantification of acrosomal responsiveness to A23187 in fertile and subfertile stallions. Flow cytometric analysis revealed that semen of the

subfertile stallions showed no significant response to A23187. This assay was able to repeatably demonstrate acrosomal dysfunction in 4 stallions with unexplained subfertility. These results support the value of acrosomal assessment when predicting the fertility of stallions, and justify the inclusion of an acrosomal reactivity test on sperm when performing a breeding soundness exam.

The fixation-permeabilization sequence in our protocol could induce an increased level of membrane damage, creating false positives for acrosome-reacting sperm. Debris that mimic sperm and bind PSA could also create a false sense of acrosome intact cells. Artifacts produced by clumping of liberated acrosomal contents pose a potential inaccuracy of fluorescent measurement as well. Fetal bovine serum was used in this protocol. Serum contains flavins and other fluorescent molecules, making it highly fluorescent [105]. Despite the above concerns, employment of flow cytometry can reduce false positives of other fluorescence-based techniques when detecting acrosome reactions of stallions by following strict gating procedures. Gating permits the evaluation of cells restricted to the parameters set by the operator. In this study, acquisition of fluorescence intensities was limited to objects that resembled stallion sperm, i.e. forward and side scatter. Even so, the development of an assay that utilizes less membrane-invasive techniques could enhance the efficiency of measuring acrosomal response in semen.

In summary, the flow cytometric procedure provided a rapid analysis of acrosomal status and acrosomal response to the potent calcium ionophore, A23187. Demonstrating a continuum of fluorescence loss as the acrosome reaction proceeded., F low cytometry is a technique that can be used to assess acrosomal function and has the potential to monitor multiple markers of sperm function. The cause of acrosomal dysfunction in sperm from some stallions is unknown, but may be associated with membrane alterations and/or disruptions in specific cellular systems. Studies that target the source of acrosomal dysfunction and the creation of a more suitable storage environment for sperm will augment management and therapeutic strategies for breeding stallions.

FUTURE AIMS

Potential future studies include: 1) the deletion of paraformaldehyde fixation, permitting the ethanol to act as the fixative as well as permeabilize the plasma membrane and 2) experiment with other semen extenders which might cause less damage to the spermatozoa. 3) Development of a protocol that would allow a time course evaluation of fluorescence intensities of sperm within the first minutes of A23187 exposure. 4) If there is an increase in forward and side scatter in the beginning stages of the acrosome reaction, these changes could be used as an indicator for the ability of the sperm to be able to acrosome react without labeling the sperm. The use of a flow cytometric sorter could provide a higher quality of semen sample from subfertile stallions for artificial insemination.

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APPENDIX

Density gradient preparation

A 40/80 EquiPure™ 100 gradient is prepared as follows:

1. Two milliliters of 40% EquiPure™ 100 carefully layered over the 2mL 80% layer to form the discontinuous gradient; set aside to allow separation of any mixed particles.
2. Extend semen 1:1 in E-Z Mixin™ CST then centrifuge for 17 minutes at 400Xg in 50mL conical vials.
3. Remove supernatant, leaving approximately 0.4cc of the sperm pellet.
4. Resuspend the sperm pellet in 1mL CST supplemented with modified Tyrode's extender (KMT) (Padilla and Foote 1991;Rigby et al. 2001).
5. Pipette extended semen sample on top of density gradient that was prepared in step 1.
6. Centrifuge for 30 minutes at 200 x g.
7. Remove the density gradient supernatant, being careful not to disrupt the pellet.
8. Combine the remaining pellet with 1mL KMT [doesn't have to be KMT if you put approx 5% seminal plasma in the extender to which the semen is to be re-extended. If you use KMT in this appendix, you must provide the composition of the extender] extender in 1.8mL centrifuge vial and centrifuge for 4 minutes at 320 x g.
9. Aspirate supernatant and re-suspend pellet with 150µL KMT extender.

The sperm from the density gradient provided a uniform acrosome population free of debris and detritus sperm that was used to define the acrosome intact region.

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May 2001